FORM PTO-1390 (REV. 12-97) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
3898US	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	09/214009

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL97/00354	24 June 1997 (24.06.97)	25 June 1996 (25.06.96)

TITLE OF INVENTION: VACCINE COMPRISING ANTIGENS BOUND TO CARRIERS THROUGH LABILE BONDS

APPLICANT(S) FOR DO/EO/US: Nico Johannes Christiaan Maria BEEKMAN, Wilhelmus Martinus Maria SCHAAPER, Kristian Dalsgaard, Robert Hans Meloen

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [XX] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [XX] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [XX] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [XX] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [XX] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [XX] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [XX] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [XX] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:

    International Search Report
    Preliminary Examination Report

U S APPLICATION NO (if known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO PCT/NL97/00354	ATTORNEY'S DOCKET NUMBER 3898US

17 [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Search Report has been prepared by the EPO or JPO $930.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) $720.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $790.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1070.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $98.00

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 930.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [ ] 20 [XX] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	22 - 20 =	2	x $22.00	$ 44.00	
Independent claims	4 - 3 =	1	x $82.00	$ 82.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1,186.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28). +				$	
SUBTOTAL =				$ 1,186.00	
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 1,186.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 1,186.00	
				Amount to be refunded:	$
				charged:	$

a. [XX] A check in the amount of $ 1,186.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 20-1469. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Laurence B. Bond
NAME

30,549
REGISTRATION NUMBER

98 DEC 30 AM 9:51

09/214009

300 Rec'd PCT/PTO 23 DEC 1998

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Nico Johannes Christiaan Maria Beekman et al.

Serial No.: PCT/NL97/00354

Filed: 24 June 1997

For: VACCINE COMPRISING ANTIGENS BOUND TO CARRIERS THROUGH LABILE BONDS

Examiner:

Group Art Unit:

Attorney Docket No.: 3898

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL248225737

Date of Deposit with USPS: 23 December 1998

Person making Deposit: Jared Turner

PRELIMINARY AMENDMENT

Box
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the above referenced application as indicated below:

IN THE CLAIMS:

In claim 3, line 1 please delete "or 2".

5. (Amended) A vaccine according to [any one of claims 1-4] claim 1 in which the fatty acid is palmitic acid.

In claim 6, line 1 please delete "or 5".

In claim 6, line 2 please substitute ----consisting essentially------ for "essentially consists".

In claim 7, line 1 please delete "or 5".

In claim 7, line 2 please substitute ----consisting essentially---- for "essentially consists".

In claim 8, line 1 please delete "or 5".

In claim 8, line 2 please substitute ----consisting essentially---- for "essentially consists".

9. (Amended) a Vaccine according to [any one of claims 1-4] claim 1 in which the antigen is a peptide and the carrier compound is another copy of said peptide coupled to a fatty acid.

In claim 11, line 1 please delete "or 10".

In claim 11, line 2 please substitute ----consisting essentially---- for "essentially consists".

12. (Amended) A vaccine preparation according to [any of claims 1-11] claim 1 together with [pharmacautically] pharmaceutically acceptable compound or adjuvant.

In claim 15, line 1 please delete "or 14".

In claim 18, line 1 please delete "or 17".

In claim 21, line 1 please delete "or 20".

22. (Amended) An immunogenic preparation obtainable by a method according to [any of claims 19-21] claim 19.

REMARKS

The Office is respectfully requested to enter the above amendment prior to the calculation of the filing fee in this application.

Respectfully submitted,

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Date: December 23, 1998

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(d)] -- NONPROFIT ORGANIZATION

P-2258 U 500

Applicant or Patentee: N.J.C.M. Beekman et al. Docket No.
Serial or Patent No.: 09/214,009
Filed or Issued: 23 December 1998
For: Vaccine comprising antigens bound to carriers through labile bonds

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Stichting Instituut voor Dierhouderij en Diergezondheid
ADDRESS OF ORGANIZATION: Edelhertweg 15, 8219 PH Lelystad, the Netherlands

TYPE OF ORGANIZATION

[ ] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) and 501(c) (3)]
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OR THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)
[X] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) and 501(c) (3)] IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE)
(CITATION OF STATUTE)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) or (b) of Title 35, United States Code with regard to the invention entitled Vaccine comprising antigens bound to carriers through labile bonds

by inventor(s) N.J.C.M. Beekman, W.M.M. Schaaper, K. Dalsgaard & R.H. Meloen

described in

[ ] the specification filed herewith
[X] application serial no. 09/214,009, filed 23 December 1998
[ ] Patent no. , issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

NAME
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME
ADDRESS
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC §1001, and may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING C.J.G. Wensing
TITLE IN ORGANIZATION General director
ADDRESS OF PERSON SIGNING Edelhertweg 15, 8219 PH Lelystad, the Netherlands

SIGNATURE DATE 5 February 1999

VACCINE COMPRISING ANTIGENS BOUND TO CARRIERS THROUGH LABILE BONDS

The invention relates to the field of immunization and vaccines. By vaccination, antibody and/or cellular responses are elicited in the vaccinated animal or human. These responses are directed against the antigen or antigens that are used in the vaccine. It has long been known that the antigenicity and immunogenicity of antigens may vary, depending for example on the way the antigen is presented to the vaccinated animal, or on the size and structure of the antigen molecule.

A widely known way of increasing the immunogenicity of a vaccine preparation is the addition of adjuvants to this preparation. However, such adjuvants stimulate the immune response only in an aspecific way, and do not increase the specific antigenicity or immunogenicity of the antigen used. Enhancement of specific antigenicity or immunogenicity of antigens that are themselves poorly antigenic or immunogenic is often achieved by coupling the antigen to carrier compounds. Synthetic peptides have frequently been used to immunize animals. Peptides of less then about 20 to 30 amino acids are, however, notorious examples of such poor immunogens. To increase their specific antigenicity or immunogenicity it is assumed that the molecular weight needs to be increased.

Many attempts have been made to conjugate antigens in a stable bond to carrier molecules, using different carrier compounds such as the carrier proteins keyhole limpet haemocyanin (KLH) or ovalbumin (OVA), in order to increase the molecular weight. Such immunoconjugation may lead to very complex structures, and generate unwanted side-effects, in the sense that e.g. unwanted antibodies are elicited that are specifically directed against the carrier compound and the areas of the conjugated molecule

where the linkage of the antigen with the carrier have taken place.

Other methods that have been applied are the coupling of fatty acid groups to proteins or peptides of low antigenicity or immunogenicity. The main purpose of the introduction of fatty acids into antigens has been to anchor hydrophillic antigens on adjuvant- and immuno-presentation systems, e.g. liposomes and iscoms, because such systems require the presence of hydrophobic molecules to allow their incorporation into these systems. Such procedures of vaccine preparation have been used with some success with model protein antigens and with less success with synthetic peptides. Acylation of the lysines of ovalbumine with palmitic acid was found to enhance the major histocompatibility complex (MHC) class II-restricted presentation. This suggests that conjugation with longer fatty acids would lead to the formation of lipopeptide T-cell epitopes with increased affinity for binding to MHC class II and/or T-cell receptors (20).

Such coupling of fatty acids has been achieved by acylation of proteins or peptides (26) intended for use in immunization/vaccination purposes, simply by linking the fatty acid covalently to the antigen. In these studies, predominantly palmitic acid or myristic acid have been used. For protein antigens, the introduction of 'lipid tails' (palmitylation) has been performed mainly by using palmitic acid N-hydroxysuccinimide, leading to an irreversible and stable bond between the protein and the fatty acid. For synthetic peptide antigens, the most common procedure is performed after synthesis of the peptide, by adding palmitic acid to the free amino group in the terminal amino acid by continuing peptide synthesis chemistry. This leads to an irreversible amide bond between the palmitic tail and the peptide.

We have now found a procedure which, instead of coupling the antigen irreversibly to a carrier compound, allows for coupling between the antigen (be it protein or

peptide or carbohydrate or any other molecule to be used as an antigen for immunization/vaccination procedures) and the carrier compound in a reversible and labile way, with a so-called labile-linking method. With a 'labile-link' a labile chemical bond between antigen and carrier protein is meant. Labile should be understood as either chemically or enzymatically labile. A chemically labile bond cleaves for example under conditions normally found in the body, e.g. at basic pH such as can be found in certain body tissues, as at acidic pH which can be found in other tissues or certain cell compartments, whereas an enzymatically labile bond cleaves in the presence of enzymes, such as thioesterases or esterases, which are present in bodily tissues.

Such cleavages result in a dissociation of the antigen and the carrier compound, after administration of the vaccine. In this way, as will be demonstrated in detail in the experimental part of this description, surprisingly a better immune response can be elicited by an in itself poorly immunogenic antigen than by methods that provide a stable link between the antigen and carrier compound.

The invention not only allows for the introduction of carrier compounds, such as lipid tails, into antigens but also allows for a better routing to the cell surface of antigen presenting cells, after which the antigen becomes dissociated and may be better processed by those antigen presenting cells, resulting in a much better immune response. Therefore, the invention is not simply aimed at anchoring antigens to carrier proteins, adjuvants, or other presentation systems, but is in its own right capable of greatly increasing the antigenicity or immunogenicity of antigens.

A theoretical explanation which should not be seen as restricting the invention is that antigens with reversible bonds or labile links to carrier compounds, for instance by palmitic acid acylation of thiol groups in peptides,

would facilitate the routing of these antigens to various cell compartiments and enhance in this way their immunogenicity.

Peptides have been substituted with palmitic acid residues in quite a number of ways: by conjugation with Nα,Nε-dipalmitoyllysine peptides (8,5), with Nα-palmitoyl-S-(2,3-*bis*-palmitoloxy-(2RS)-propyl)-(R)-cysteine (Pam_3Cys-peptides (5,13)), palmitoylated polylysine (23,24), Pam_3Cys-Multiple Antigen Peptides (4,10), lipo-Multiple Antigen Peptides (9,10), N-palmitoylated proteins used for incorporation into ISCOMs (3,14,17), and peptidyl-Nε-palmitoyl-lysines (5). In these examples, palmitoylation was at an amino group or a hydroxyl group, providing irreversible bonds such as amides.

The present invention as exemplified in the experimental part, which utilizes the formation of a labile bond, now provides a very simple approach to obtain labily linked antigen and carrier compound via the controlled conjugation of the antigen with one long-chain fatty acid. The presence of the fatty acids increases the uptake of the antigen into antigen presenting cells, and, due to the labile or reversible nature of the bond, the antigen becomes separated again, and specific antibody formation directed to the antigen only is elicited.

In the experimental part the concept of labile linking is demonstrated with synthetic peptides as antigen linked via a thioester bond to palmitic acid as carrier compound or with a synthetic peptide as antigen linked via a disulfide bond to a N-palmitoyl peptide as carrier compound. A preferred part of the invention is thus illustrated by the examples provided. However, any vaccine or immunogenic preparation in wich the antigen is reversibly or instabily or labily bound to the carrier compound by whatever labile link imaginable with the purpose that the antigen dissociates from the carrier compound after administration of the vaccine is also part of the invention. For example, a vaccine composition

comprising a glycopeptide, linked via a disulfide bridge to ovalbumine, will dissociate as soon as the composition encounters reducing circumstances, as for instance can occur in the blood in the presence of glutathion.

As antigen, other types of molecules to which an immune response preferably needs to be elicited, such as polypeptides, proteins with or without carbohydrate moieties or other side chains, carbohydrate chains themselves, nucleic acid molecules, haptens, etc., can be chosen. As carrier compound, other fatty acids can be used, but also carrier proteins, such as KLH or OVA, or fatty acid peptides, or other types of molecules can be used as carrier compound.

For the labile link between the antigen and the carrier compound, the thioester bond illustrates a preferred part of the invention. However, other chemical bonds such as esters and disulfide bonds that are labile under physiological conditions (such as pH or osmolarity or salt concentrations or the presence of reducing agents or enzymes) which are usually present in body fluids or in or on body tissue or cells also fit the purpose of providing a vaccine or immunogenic preparation in which the antigen dissociates from its carrier compound under physiological conditions, i.e. after it has been administered. Typically good examples of labile linkers can be found in the field of tumortherapy where such linkers have been applied between antibodies and cytostatic agents.

Also, vaccines and immunogenic preparations that are prepared according to the invention can be mixed with carrier compounds and adjuvants that are traditionally used to render antigens more antigenic or immunogenic. In addition, vaccines and immunogenic preparations that are prepared according to the invention can be mixed with other compounds or adjuvants or antigen delivery systems that are specifically chosen to hasten or slow the rate of dissociation of the antigen from the carrier compound

after the preparation has been administered, i.e. by surrounding the preparation with conditions in which the dissociation rate is altered, and thereby shielding it temporarily from normal physiological conditions. For sustained release of the antigen from the carrier it may also be useful to apply different linkers having different dissociation constants between carrier and antigen.

Furthermore, vaccines and immunogenic preparations that are prepared according to the invention can be administered in various ways known in the art, for instance by but not limited to intramuscular, intranasal, intraperitoneal, intradermal, intracutanous, mucosal or aerosol application of the preparation.

EXAMPLES

Introduction

The concept of labile linking is demonstrated with synthetic peptides as antigen and palmitic acid or a N-palmitoylpeptide as carrier compound.

Conditions for acylation of peptides in solution or on solid phase were established. Furthermore, it was demonstrated that the stability of the bond influences the immunological efficacy, because in our study conjugation of a fatty acid implies either formation of a stable amide when N-acylation is involved, or formation of a labile thioester in the case of S-acylation. It was found that this difference in site of acylation (N or S) is important with respect to its immunogenicity. Upon immunization it was found that labile S-palmitoylated peptides or peptides coupled to a N-palmitoylated peptide via a labile disulfide bridge are superior to N-palmitoylated peptides and at least comparable to KLH conjugated peptides with respect to response time of detectable antibody formation or biological effect. A theoretical explanation is that, under biological or physiological conditions, the presence of appropriate fatty acid chains chemically linked through

labile thioester or disulfide bonds, improves immunogenicity, probably because it represents a favourable substrate for take up and processing in cells of the immune system. This processing can be mediated by palmitoyl thioesterases, that have been isolated (18), or for disulfides by glutathion.

Unlike amides and esters, thioesters are readily susceptible to nucleophilic attack. The chemical stability of palmitoyl thioesters had thus to be considered at physiological pH and during deprotection conditions (acid, acid-thiol, base). Nine peptide constructs (A, B, C, D, E, F, G, H and I, Fig. 1) were prepared as vaccine preparation, two peptides represent a GnRH-tandem peptide, five are based on the N-terminus of VP2 (positions cys-2-21) of canine parvovirus (CPV (11)), and two are from the V3 loop of feline immunodeficiency virus (FIV). They differ by their acylation sites (S, α-N or ε-N, Fig. 1). It was found that S-palmitoylated peptides induce high levels of antibodies, comparable to or slightly better than KLH-MBS linked peptides, and superior to N-palmitoylated peptides with respect to response time of detectable antibodies (CPV) or biological effect (GnRH).

MATERIALS:

N-methylpyrrolidone (NMP), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) and piperidine were peptide synthesis grade and obtained from Perkin Elmer/ABI (Warrington, UK).
Dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF), N-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), acetonitrile (ACN), trifluoroacetic acid (TFA)

(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (Rink resin) (19)) were obtained from Saxon Biochemicals (Hannover, Germany). 4-methylbenzhydrylamine (MBHA) resin was obtained from Novabiochem, Laufelfingen, Switzerland. For analytical HPLC we used two Waters pumps model 510, a Waters gradient controller model 680, a Waters WISP 712 autoinjector, and a Waters 991 photodiode array detector. Products were analyzed in a linear gradient from water with 0.1% TFA to 60% acetonitrile/water with 0.1% TFA in 60 minutes on a Waters Delta Pak C_{18}-100 (3.9x150mm, 5µm) column at 1 ml/min.

Amino acid analysis was performed using a Waters Pico-Tag system, after hydrolysis in a Pico-Tag workstation using 6N HCl at 150°C for 1 hour, and derivatization with phenylisothiocyanate. Preparative HPLC was carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cardriges plus guard cardridge (40x210 mm or 25x210 mm) filled with Delta-Pak C_{18}-100 (15 µm) material. Peptides were detected at 230 nm using a Waters 486 spectrophotometer with a preparative cell.

METHODS:

1) Synthesis of peptides.

All peptides (except peptide B: Fig. 1) were synthesized as C-terminal amides on a Rink resin (loading 0.38 mmol/g), using the FastMoc™ method (7) on an ABI 430A peptide synthesizer. Cysteine sulphur was protected with a S-tert-butylsulfenyl group or a trityl group, all other side chain functions were protected with trifluoroacetic acid labile groups. After completion of the synthesis, peptides were either acetylated using acetic anhydride/DIEA/NMP (0.1/0.01/1 (v/v/v)) or N-palmitoylated using pre-activated palmitic acid (see Methods; sections 3 and 4). Peptides with S-trityl protected cysteine were cleaved from the resin and concomitantly deprotected by acidolysis in the presence of

scavengers using TFA/water/EDT/TA/phenol (40/2/1/2/3) (v/v/v/v/w)) for 2 hours. The crude peptides were precipitated and washed twice with hexane/ether (1:1) and lyophilized from acetonitrile/water (1:1). Peptide B was synthesized as C-terminal amide on a methylbenzhydrylamine (MHBA, 0.46 mmol/g) resin, starting with Fmoc-Lys(Boc)-OH in NMP. After cleavage of the Boc group by TFA/water (19:1), lysine was palmitoylated with pre-activated palmitic acid (see Methods; section 4). After Fmoc deprotection, the α-amino groups were successively acylated as described above with the pertinent Fmoc-amino acids to give the protected peptide. Compound B was deprotected and cleaved from the resin by HF/anisole (9:1).

2] Deprotection of S-(tert-butylsulfenyl)cysteinyl residues in solution.

The side chain protection of cysteine was removed using a reducing agent: a double deprotection of 30 minutes was routinely used, each with a 50 eq. molar excess tri(n-butyl)phosphine in 10% H_2O/NMP under nitrogen atmosphere. The reaction was monitored by HPLC analysis.

3] S-palmitoylation in solution.

-*Activation of palmitic acid*. To a solution of palmitic acid (100 μmol) in DCM (250 μl) was added 100 μmol of HBTU/HOBt in DMF (0.45M, 220 μl) and 200 μmol 2M DIEA in NMP. The activated palmitic acid formes a gel from this mixture in about 20 minutes at room temperature.

-*Palmitoylation of a thiol group*. An acetylated peptide (not containing lysyl residues) was dissolved in NMP at a concentration of 10 mg/ml. The solution was brought to pH 5 with DIEA (2M in NMP), as checked by using wet pH paper, and mixed with one eq. of pre-activated palmitic acid. After 90 min at room temperature and stirring, a sample was subjected to analytical HPLC. About 50% of the peptide was found to have reacted. A second

equivalent of pre-activated palmitic acid, this time prepared with only half the amount of DIEA, was added and after another 90 min more than 70% of the peptide was palmitoylated and no by-products were found. After a total reaction period of 5 hours, the mixture was diluted fourfold with acetonitrile/water (1:1), two drops of TFA were added and the palmitoylated peptide was isolated and purified by preparative HPLC.

-Palmitoylation of an α- or an ε-amino group. A free Nα or a free Nε of a lysyl residue were N-palmitoylated as decribed for S-palmitoylation.

4] S-palmitoylation on the resin.

After completion of the synthesis, the resin was treated twice for 60 minutes (or the second time overnight, depending on the quality of the phospine) with tri(n-butyl)phosphine (50 eq.) in NMP/water (9:1) to remove the tert-butylsulfenyl group. The resin was washed copiously with NMP. Free SH groups were palmitoylated on the resin by double coupling using the pre-activated palmitic acid reagent (see palmitoylation in solution, section 3) in DCM/NMP for 5, resp. 16 hours.

5] Selection of deprotection mixture and stability of thioester with respect to acid.

To investigate the stability of thioesters during acidolytic removal of tert-butyloxy functions (Boc and OBu^t) and/or cleavage of the resin-peptide bond, two model peptides, Ac-C(palm)SEIFRPGGGDMR-NH_2 and Ac-C(palm)VATQLPASF-NH_2, both not containing lysine, were palmitoylated in solution and purified with preparative HPLC. Samples were then subjected to the conditions of acidolysis in the presence or the absence of scavengers using TFA/water (19/1 v/v) (**A**), TFA/water/EDT/TA/phenol (40/2/1/2/3 v/v/v/v/w) (**B**), or EDT/water/TFA (1/1/38 v/v/v/) (**C**). Acetic acid/water (1/1) was used as a control. The treatment (3 hours) was at room temperature.

The thioester proved to be stable under all these acidic conditions.

6] Blocking of free SH groups by iodoacetamide.

To prevent the peptide ac-CSDGAVQPDGGQPAVRNERATG-NH_2 from dimerization by air oxidation, the free SH group was blocked. Peptide ac-CSDGAVQPDGGQPAVRNERTAG-NH_2 (20 µmol) and iodoacetamide (100 µmol) were dissolved in 400 µl DMF and 600 µl 2% NH_4HCO_3 (pH 8.0). The pH was kept between 7.5 and 8.5, and if necessary corrected with extra NH_4HCO_3 (grains). The solution was stirred for some 1.5 or 2 hours at room temperature. Acetic acid (100%, 50 µl) was added to stop the reaction. An Ellmann test was used to analyse the result.

7] Stability of the thioester with respect to base.

The stability of peptide A was investigated in phosphate buffered saline (PBS, pH 7.2)/DMF (7/3), aqueous NH_4HCO_3 (2%, pH 8)/DMF (7/3) piperidine/NMP (3/7). Peptide A was dissolved at a concentration of 5 mg/ml, at room temperature. The stability was monitored by analytical HPLC (Table 1). The results are summarized in table 1.

8] Synthesis of disulfide construct C of CPV

ac-CSDGAVQPDGGQPAVRNERATG-NH_2 (13 mg) and palm-CSDGAVQPDGGQPAVRNERATG-NH_2 (6.4 mg) were dissolved in 1 ml of 50% DMSO/DCM. The solution was brought to pH 7.0 with 150 µl of 2% NH_4HCO_3. The mixture was stirred and the reaction was followed by analytical HPLC. After 3 hours the reaction was stopped by the addition of 0.5 ml of acetic acid. The heterodimer C (Fig. 1, disulfide) and the homodimer of the acetyl peptide were isolated by HPLC purification. To obtain an optimal yield of compound C equimolar quantities of both starting peptides can be used. The dimerization can also be performed in 10% DMSO/NMP using triethylamine for neutralization. The molar mass was determined by FAB-MS. Since reducing thioglycolic

acid was added for mass analysis, only the two single chain peptides were found (ac-peptide: calc: 2422, found 2422 and 2489; palm-peptide: calc: 2465.79, found 2465,20). Two side reactions, that occured during synthesis, were responsible for a number of side products (26): an extra mass of 67 was caused by a reaction of piperidine with one of the aspartic acid residues, losing one molecule of water, and a loss of 18 was caused by an Asp-Gly rearrangement (DG occurs twice in the sequence!), a side reaction that frequently occurs especially in DG sequences. Electrospray MS (ES-MS) revealed the mass of the complete construct C, but also the masses of the construct C modified by the side reactions mentioned above. These modifications will not influence the immuneresponse significantly.

9] Conjugate preparation.

Keyhole limpet haemocyanin (KLH, 10 mg, Calbiochem, La Jolla, CA) was dissolved in 0.1 M sodiumphosphate buffer, pH 7.0, at a concentration of 12.5 mg/ml. Acetonitrile (0.3 ml) was added, followed by 100 µl of 0.125 M maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Pierce, Rockford, IL) solution in DMF. After stirring for 1 hour, the solution was dialysed three times for 30 minutes against 0.1 M phosphate buffer, pH 7.0 at 4°C. Peptide (10 mg) was added to the modified KLH and the mixture was shaken overnight at room temperature. The conjugate was dialyzed two times for 2 hours and then overnight against 0.1 M phosphate buffer, pH 7.0 at 4°C.

10] Vaccine formulation.

For each vaccine, peptide constructs (conjugated to KLH, N- or S-palmitoylated) were dissolved in phosphate-buffered saline (PBS, pH 7.2) and emulsified with complete Freund's adjuvant (CFA) in equal volumes. Mixtures were emulsified by repeatedly forcing it through a needle until a stable water-in-oil emulsion was obtained. The emulsion

was prepared just before immunization. Second immunizations were prepared with incomplete Freund's adjuvant (IFA).

11] Vaccination or immunization.

GnRH-tandem constructs. Six groups of 7-8 male piglets at the age of 10 weeks were immunized with compound A or B (Fig. 1). Each animal was injected with 1 ml of vaccine formulation (1, 0.25 or 0.05 mg) administered intramuscularly in the neck. The second immunization was given 8 weeks later. One group served as a control and was mock immunized with only CFA and KLH. At week 26 the animals reached their slaughterweight; their testicles were excised, epididymes were removed and testes weight was recorded.

CPV constructs. In the first experiment, two groups of 5 guinea pigs were immunized with compound C or D (Fig. 1). Each animal was injected with 500 µl vaccine formulation administered subcutaneously in the neck. The second immunization was given 6 weeks later. One group served as a control and was immunized with compound F (KLH-MBS coupled peptide construct, Fig. 1). Blood was taken at weeks 0, 6, 7 and 12 post immunization and anti peptide antibody titers were recorded in an ELISA.

In the second experiment, one group of 3 guinea pigs was immunized with compound C emulsified in CFA. For comparison, compound E was emulsified in CFA. Blood was taken at 0, 4, 8 and 16 weeks post immunization and anti peptide antibody titers were recorded in an ELISA.

In the third experiment, three groups of three guinea pigs were immunized with compound D, E or F. CFA was used to emulsify the peptides. As a control, the free peptide G was used in which the thiol group was blocked with iodoacetamide to prevent dimerization of the peptide. Blood was taken at 0, 4 and 8 weeks post immunization and anti peptide antibody titers were recorded in an ELISA.

FIV constructs. In this experiment six groups of four cats were immunized with compound H (in IFA or in ISCOMS) or with compound I (conjugated with cholera toxin via MBS, given either subcutaneously, rectally, or intranasally). Blood samples were taken at 8 weeks post vaccination and antibody titers against the peptide were determined in an ELISA.

Results

1) Methodological.

A method has been developed for the acylation of peptide thiols in solution or within the matrix of the solid support. In solution, peptides with only one single nucleophilic function (N or S) can be acylated in a very efficient way using pre-activated palmitic acid. S-acylation of protected peptides on the solid support requires an efficient and selective removal of the *tert*-butylsulfenyl protection, which was realized effectively by reduction with tri(n-butyl)phosphine in NMP/water (9/1). An advantage of this approach is that it can be done in the presence of lysines, because the lysines are still protected. The thiol palmitates (A and E; Fig. 1) were obtained by acylation in solution with a pre-activated palmitic acid; B, E and the palmitoyl peptide of compound C were palmitoylated on the resin. Thioester peptides were found to be stable during acidolysis of other protective functions. The palmitoyl-thioester bond is not stable under basic conditions. Buffered solutions of the thioesters around pH 8 should not be left standing for prolonged periods (Table 1).
At pH 7, stability is already much better and lyophilized thioester at -18°C was found to be completely stable after 28 months.

2] Immunogenic potency of palmitoylated peptides.

a] GnRH-tandem peptides A and B:

The immunogenic potency of the synthetic GnRH tandem peptides A and B was tested in an experiment on six groups of 7-8 male piglets (age 10 weeks), each group recieving a dose of either S-palmitoylated (A) or N-palmitoylated (B) antigen. The GnRH tandem peptides A and B exhibited different immunogenic effects, as observed on testis weight (15) at the age of 26 weeks after two injections in the presence of CFA or IFA (second injection, see Table 2). While both forms were able to keep testis size low, only the S-palmitoylated peptide A was able to do this in all animals at the highest dose tested (1 mg), and still in 3 out of 8 animals at the lowest dose (0.05 mg) tested. The response was also dose dependent. A dose of 0.25 mg S-palmitoylated peptide A gave a similar effect as a dose of 1 mg N-palmitoylated peptide B.

b] CPV peptides:

The above observed effect with the palmitoylated GnRH peptides A and B was further investigated with another construct, and in another animal species. In following experiments, the N-ternimal fragment of VP2 (positions 2-21) of canine parvovirus was used. This peptide, when conjugated via a disulfide bridge to the same peptide with a N-palmitoyl group (peptide construct C), appeared to be capable to elicit antibody responses as well as the peptide conjugated to KLH (peptide F, etc.). On the other hand, the N-palmitoylated peptide alone (peptide D, probably partly present as dimer) did yield a lower immunogenic effect, since antibody titers were lower and did not develop in all animals (see Table 3). Peptide construct C was formed during first synthesis of peptide E. In this synthesis we coupled Fmoc-Cys(StBu)-OH to the parvo peptide-resin, then cleaved the StBu side chain protecting group of cysteine, using 2-mercaptoethanol for 48 hours. This step also partially cleaved the Fmoc group.

The resin was washed and the free cysteine was palmitoylated at the thiol group and partially at the amino group as described. Next, the Fmoc group was cleaved using piperidine. In this step the palmitoyl thioester was also cleaved and partially shifted to the free amino group of cysteine (S⇒N shift). Then, the peptide was acetylated and the peptide was deprotected and cleaved from the resin as described. After workup and purification by preparative HPLC, product C, a synthetic peptide linked with a disulfide bond to a fatty acid linked to another copy of said synthetic peptide, was isolated together with some minor side products. Mass spectrometry showed that peptide construct C was partially rearranged (26), because of the Asp-Gly sequence that occurs twice in the parvo peptide. However, the addition of a piperidyl group to aspartic acid at position 3 or 9, or the loss of water molecule by the formation of an aspartimide will not influence the immune response significantly. A second experiment was performed in which peptide E was compared with peptide construct C in CFA. In this synthesis of peptide E, the peptide was acetylated directly after the coupling of Fmoc-Cys(StBu)-OH, thus preventing palmitoylation of the amino group. After cleavage of the StBu group, S-palmitoylation, and final deprotection, the parvo thioester product was purified using HPLC. This resulted in a product E, a synthetic peptide linked with a thioester bond to a fatty acid, where no piperidine adducts were detected by analytical HPLC and mass analysis. Product E and product C had a similar immune response (see table 4). The superiority of the S-palmitoylated product above the N-palmitoylated peptide was further confirmed by direct comparison of peptide D, peptide E, and the KLH-MBS-conjugated peptide F (Table 5). In this *third experiment* the antibody titer elicited with the S-palmitoylated peptide at 4 weeks past vaccination was almost 100 times higher than the titer obtained with the N-palmitoylated peptide. At week 8 the titers were

almost equal. Compared to the KLH-conjugated peptide, the titers for the S-palmitoylated peptide were both in the 4th and the 8th week higher.

C] FIV peptides:

The antibody response of the palmitoyl-thioester peptide H, either in IFA or in ISCOMS was much higher than the response against the cholera toxin conjugated peptide. The way the CT peptide conjugate was administered could not improve the responses much (see Table 6).

Discussion of experimental results in the examples

Immunogenic aspects.

In this study, procedures for palmitoylation through an amide or thioester bond were established. Furthermore, we investigated the immunogenic potency of peptides conjugated to a fatty acid, and compared it with KLH-MBS-conjugated peptides. It was found that the site of acylation (N or S) effects the immunogenicity of the adduct. Surprisingly, palmitoylation of the peptide through a thioester bond (S acylation) yields a highly immunogenic product, being as potent as or even better than a peptide coupled to a carrier protein (KLH, OVA or CT). On the other hand, N-palmitoylation is far less immunogenic compared to S-palmitoylation or KLH-MBS conjugation. A peptide conjugated via a labile disulfide bond with an N-palmitoylated peptide carrier, however, also induces high antibody responses. Thus, a reversible and labile bond between the antigenic peptide and a carrier molecule, being palmitic acid or a palmitoylated peptide, drastically enhances the immune response. The introduction of a fatty acid through a thioester bond thus obviates the necessity of a large carrier protein. The conventional conjugation with a large molecule (a protein) induces difficulties because the chemistry underlying the coupling is not always well controlled, while in addition

unwanted antibodies against carrier protein and linker are induced.

Chemical aspects:

Nα-acetylated peptide amides can be prepared automatically through solid-phase synthesis. However, removal of the base-labile Fmoc groups with piperidine precludes the use of S-palmitoylated cysteinyl derivatives during synthesis. Therefore, S-acylation had to be performed at the end of the synthesis on the resin or after cleavage and deprotection in solution. The acylation reaction applied here can not discriminate between thiols and amino groups. Since peptides upon cleavage from their support loose also their N-protecting groups, selective palmitoylation can only be performed in solution with compounds comprising one single nucleophilic function (N or S). Hydroxyl groups are not affected.

Because of the instability with respect to base it is not recommended to leave S-palmitoylated peptides in buffered (pH 7-8) solutions for prolonged periods (see Table 1). Thioesters do not dissociate in acidic media and do thus not contain thiolate anions which would attack thioesters. We confirm the general rule that thioesters resist thiols in strongly acidic media enabling the use of thiols as scavengers for carbonium ions during deprotection of *tert*-butyloxy functions (cf. 21).

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Table 1. Stability of the thioester bond in basic solutions

	2 hours	22 hours	95 hours
PBS(pH 7)/DMF (7:3)	>99%	90%	62%
2%NH_4HCO_3(pH8)/DMF (7:3)	>97%	30%	0%
piperidine/NMP (3:7)	0%	0%	0%

Results are given as percentage of the initial concentration of GnRH tandem peptide (A) as estimated by analytical HPLC after a given time period at room temperature.

Table 2. Testis weight of male piglets immunized with different doses of GnRH tandem peptides

peptide	dose (µg)	testis weight[a]		
		<100	100-150	>150
A (thioester)	1000	7/7	0/7	0/7
,,	250	6/8	1/8	1/8
,,	50	3/8	0/8	5/8
B (amide)	1000	5/7	0/7	2/7
,,	250	1/7	2/7	4/7
,,	50	0/6	0/6	6/6
control (CFA)	-	0/7	0/7	7/7

[a]: number of animals in weight category/total number of animals. Testis weights <100 gram were considered positive, between 100-150 gram intermediate and more than 150 gram negative.

Table 3: Serum anti-peptide antibody titers at 6, 7 and 12 weeks of guinea pigs vaccinated with 100 μg compound C, D or F emulsified in CFA. Booster vaccination emulsified in IFA was given at week 6 post first vaccination. Blood samples were already taken.

		titer[a]		
animal	compound	6wpv	7wpv	12wpv
1	C	5.1	6.0	>6.5
2	C	5.1	5.0	4.8
3	C	6.1	5.4	5.4
4	C	5.0	4.9	5.0
5	C	4.6	#[b]	
6	D	3.5	4.1	4.3
7	D	1.7	1.6	2.9
8	D	3.5	3.7	3.7
9	D	-[c]	-	-
10	D	2.0	2.0	#
11	F	5.1	5.3	5.7
12	F	5.0	5.0	>6.5
13	F	4.5	4.7	#
14	F	5.2	#	
15	F	5.0	5.2	5.3

[a]: zero blood samples were subtracted before calculation. The titer was calculated as the -log(dilution factor) which give a signal three times the background.

[b]: animal died during the experiment.

[c]: means no detectable titer, lowest dilution measured was 1/10

Table 4: Serum anti-peptide antibody titers after 4, 8 and 16 weeks in guinea pigs. Compound C was compared with compound E, both emulsified in CFA.

		titer[a]		
animal	compound	4wpv	8wpv	16wpv
1	C	2.2	3.7	3.8
2	C	2.1	2.4	2.8
3	C	2.2	3.8	3.8
4	E	-[b]	3.0	4.0
5	E	2.1	3.5	4.6
6	E	2.1	2.4	2.5

a: zero blood samples were subtracted before calculation. The titer was calculated as the -log(dilution factor) which give a signal three times the background.

b: - means titer lower than 2.0

Table 5: Serum anti-peptide antibody titers after 4 and 8 weeks of guinea pigs immunized with 100 μg palmitoylated peptides (S or N), conjugated (KLH-MBS) or blocked (iodoacetamide).

		titer[a]	
animal	compound	4 wpv	8 wpv
1	E	2.5	2.5
2	E	4.0	3.3
3	E	3.1	3.4
4	D	1.8	2.9
5	D	-[b]	3.4
6	D	-	2.3
7	F	2.5	2.2
8	F	2.7	2.0
9	F	-	-
10	G	-	-
11	G	-	-
12	G	-	-

[a]: zero blood samples were subtracted before calculation of the titer. The titer was calculated as the -log(dilution factor) which give a signal three times the background.

[b]: - no detectable titer, lowest dilution measured was 1/30.

Article 34

Claims

1. A vaccine comprising an antigen and a fatty acid or fatty acid peptide carrier compound which are directly or indirectly linked by a thioester or a disulphide bond that is labile and dissociates under certain physiological conditions.

2 A vaccine according to claim 1 in which said antigen dissociates from said carrier compound after the vaccine or preparation has been administered.

3 A vaccine according to claim 1 or 2 in which said antigen is a protein, a polypeptide, a synthetic peptide, a carbohydrate, or a hapten.

4 A vaccine according to claim 3 in which the antigen is a synthetic peptide.

5 A vaccine according to any one of claims 1 - 4 in which the fatty acid is palmitic acid.

6 A vaccine according to claim 4 or 5 in which the synthetic peptide essentially consists of the amino acid sequence /EHWSYGLRPGQHWSYGLRPG.

7 A vaccine according to claim 4 or 5 in which the synthetic peptide essentially consists of the amino acid sequence SDGAVQPDGGQPAVRNERATG.

8 A vaccine according to claim 4 or 5 in which the synthetic peptide essentially consists of the amino acid sequence RAISSWKQRNRWEWPRD.

9 A vaccine according to any one of claims 1-4 in which the antigen is a peptide and the carrier compound is another copy of said peptide coupled to a fatty acid.

10 A vaccine according to claim 9 in which the carrier compound is an N-palmitoylated peptide.

11 A vaccine according to claim 9 or 10 in which the peptide essentially consists of the amino acid sequence SDGAVQPDGGQPAVRNERATG.

12 A vaccine preparation according to any of claims 1-11 together with pharmacautically acceptable compound or adjuvant.

13 A composition comprising a synthetic peptide linked with a thioester bond to a fatty acid.

14 A composition according to claim 13 wherein the fatty acid is palmitic acid.

15 A composition according to claim 13 or 14 wherein the peptide is selected from the group consisting of /EHWSYGLRPGQHWSYGLRPG, SDGAVQPDGGQPAVRNERATG and RAISSWKQRNRWEWPRD.

16 A composition comprising a synthetic peptide linked with a disulfide bond to a carrier compound comprising a fatty acid linked to another copy of said synthetic peptide.

17 A composition according to claim 16 wherein the fatty acid is palmitic acid.

18 A composition according to claim 16 or 17 wherein the peptide is selected from the group consisting of /EHWSYGLRPGQHWSYGLRPG, SDGAVQPDGGQPAVRNERATG and RAISSWKQRNRWEWPRD.

19 A method for producing an immunogenic preparation comprising linking a synthetic peptide with a fatty acid or fatty acid peptide carrier compound via a thioester or disalphide bond that is labile and dissociates under physiological conditions.

20 A method according to claim 19 wherein the fatty acid is palmitic acid.

21 A method according to claim 19 or 20 wherein the peptide is selected from the group consisting of /EHWSYGLRPGQHWSYGLRPG SDGAVQPDGGQPAVRNERATG and RAISSWKQRNRWEWPRD.

22 An immunogenic preparation obtainable by a method according to any of claims 19-21.

23 A vaccine comprising an immunogenic preparation according to claim 22 together with pharmaceutically acceptable compound or adjuvant.

Rev. 1-3/11/83

Effective February 27, 1983

DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATIONS

( ) Original ( ) Supplemental ( ) Substitute (X) PCT

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: Vaccine comprising antigens bount to carriers through labile bonds

which is described and claimed in:
( ) the attached specification, or
(X) the specification in the application Serial No. 09/214,009 filed 23 December 1998; and with amendments through _____ (if applicable),
(X) the specification in International Application No. PCT/NL97/00354, filed 24 June 1997, and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
Europe	96201766.1	25 June 1996	(X) YES ( ) NO
			( ) YES ( ) NO
			( ) YES ( ) NO
			( ) YES ( ) NO
			( ) YES ( ) NO
			( ) YES ( ) NO

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

SERIAL NO.	U.S. FILING DATE	STATUS
		( ) Patented ( ) Pending ( ) Abandoned
		( ) Patented ( ) Pending ( ) Abandoned
		( ) Patented ( ) Pending ( ) Abandoned

As a named inventor I hereby appoint: P.T.O.

as my attorneys to prosecute this application and to transact all business in the United States Patent and Trademark Office in connection herewith. Direct all correspondence to: TRASK, BRITT, & ROSSA, 525 South 300 East, P.O. Box 2550, Salt Lake City, Utah 84110, U.S.A.
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P22599.1500

Effective February 27, 1983

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RESIDENCE & CITIZENSHIP	CITY Lelystad NLX	STATE OR COUNTRY the Netherlands	COUNTRY OF CITIZENSHIP the Netherlands	
POST OFFICE ADDRESS	ADDRESS Karveel 10-04	CITY Lelystad	STATE OR COUNTRY the Netherlands	ZIP CODE 8231 AP
FULL NAME OF 5TH INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP	
POST OFFICE ADDRESS	ADDRESS	CITY	STATE OR COUNTRY	ZIP CODE
FULL NAME OF 6TH INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME	
RESIDENCE & CITIZENSHIP	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP	
POST OFFICE ADDRESS	ADDRESS	CITY	STATE OR COUNTRY	ZIP CODE

I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor _____ Date 5 February 1999

2nd Inventor _____ Date 5 February 1999

3rd Inventor _____ Date 5 February 1999

4th Inventor _____ Date 5 February 1999

5th Inventor _____ Date _____

6th Inventor _____ Date _____